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New and alternative strategies for the prevention, control, and treatment of antibiotic-resistant *Campylobacter*

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Abstract

Campylobacter is an enteric pathogen and a leading bacterial cause of diarrhea worldwide. It is widely distributed in food animal species and is transmitted to humans primarily through the foodborne route. While generally causing self-limited diarrhea in humans, *Campylobacter* may induce severe or systemic infections in immunocompromised or young/elderly patients, which often requires antibiotic therapy with the first-line antibiotics including fluoroquinolones and macrolides. Over the past decades, *Campylobacter* has acquired resistance to these clinically significant antibiotics, compromising the effectiveness of antibiotic treatments. To address this concern, many studies have been conducted to advance novel and alternative measures to control antibiotic-resistant *Campylobacter* in animal reservoirs and in the human host. Although some of these undertakings have yielded promising results, efficacious and reliable alternative approaches are yet to be developed. In this review article, we will describe *Campylobacter*-associated disease spectrums and current treatment options, discuss the state of antibiotic resistance and alternative therapies, and provide an evaluation of various approaches that are being developed to control *Campylobacter* infections in animal reservoirs and the human host.

Keywords

Campylobacter; antibiotic resistance; therapeutics; control strategies

Introduction

Campylobacter, a member of *Epsilonproteobacteria*, is a major bacterial cause of foodborne diarrhea worldwide.[1, 2] According to the estimation by Kirk *et al.*, more than 95 million

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cases of foodborne illnesses were attributed to *Campylobacter* worldwide in 2010.[1] In the U.S., it is estimated that *Campylobacter* is responsible for more than 1.3 million cases of illnesses each year.[3] As an enteric and zoonotic organism well adapted in the intestinal environment of various food animal species, *Campylobacter* has a broad range of animal reservoirs and is highly prevalent in ruminants, swine, and poultry. Consequently, meat products (particularly poultry) are often contaminated by *Campylobacter* during the slaughtering process. Sporadic cases of campylobacteriosis in humans are mainly caused by consumption of undercooked poultry meat, while outbreaks are primarily related to ingestion of raw milk or dairy products.[4–6] *Campylobacter* is also carried in the intestinal tract of companion animals, and contact with *Campylobacter*-infected puppies has also been implicated in recent outbreaks in the U.S..[7] While *Campylobacter* infection generally causes mild diarrhea, severe, persistent or systemic infections (e.g. bacteremia) may occur in young children, the elderly, and patients with underlying conditions of immunodeficiency.[8] Under these circumstances, antibiotic therapies are necessary and may require prescription of antibiotics of the fluoroquinolone, macrolide or aminoglycoside classes.[4] In response to antibiotic usage in clinical settings and in animal agriculture, *Campylobacter* has developed various resistance mechanisms and consequently antibiotic-resistant *Campylobacter* is increasingly prevalent, threatening the effectiveness of antibiotic therapies and posing a serious concern for public health.[9, 10] Because of the concern, antibiotic-resistant *Campylobacter* has been designated as one of the high-priority pathogens in the WHO list for development of new antibiotic therapies.[9, 10] The significance of *Campylobacter* as a major enteric pathogen and as an antibiotic resistance risk of high priority has heightened the need for developing novel and alternative approaches to combat infections caused by antibiotic-resistant *Campylobacter*. In this review article, we will present a synopsis of *Campylobacter*-associated clinical diseases in humans and animals, discuss the current state of antibiotic resistance and alternative antibiotic therapies, and provide an evaluation of new and potential approaches that are being developed for prevention, control, and treatment of *Campylobacter*.

Clinical diseases and treatment options in humans

Although *Campylobacter jejuni* and *Campylobacter coli* cause majority (95%) of the clinical diseases, close to 15 other *Campylobacter* species have been identified from human infections.[11] The infective dose of *C. jejuni* in humans can be as low as 500 organisms and the mean incubation period is around 3 days.[12, 13] Abdominal pain and diarrhea are present in > 80% of the patients, whereas fever, myalgia and headache occur in about half of the patients. A smaller proportion (10–15%) of patients also report vomiting and blood in the feces. The onset may be abrupt with diarrhea, which is usually profuse and watery, or it may be preceded by a prodromal phase of flu-like symptoms. Typically, within 4–7 days the diarrhea began to cease, however some patients can continue with the diarrheal phase for up to 2 weeks. In addition to the enterocolitis, extra intestinal manifestations in humans include abscesses, meningitis and bacteremia.[14, 15] These conditions happen more commonly in immunocompromised, pregnant and elderly patients.

Although the disease is self-limiting in the majority of the cases, antibiotic treatment using a fluoroquinolone or macrolide is becoming increasingly frequent, with a recent analysis

suggesting that up to 80% of individuals in the community receive an oral antibiotic, predominantly a 3–5 day course of a macrolide antibiotic such as azithromycin.[16] The use of fluoroquinolone antibiotics has led to the development of resistance, and a 75–90% prevalence of fluoroquinolone resistance has been reported in clinical *Campylobacter* strains in different countries.[17–19] Thus, macrolides are now the first-line treatment of human campylobacteriosis. However, the rising macrolide resistance rates, especially in *C. coli* strains from China, Spain, and Peru, have raised concerns around using macrolides as first-line treatment in those settings.[20, 21]

In addition to the acute morbidity, chronic sequelae are frequently reported following *Campylobacter* infection in humans. Post-infection irritable bowel syndrome (PI-IBS), characterized by chronic abdominal pain and bowel disturbances, has been reported to develop in ~14% of patients suffering from *Campylobacter* enterocolitis with an odds ratio of 4 compared with uninfected controls from the same population.[22] Symptoms of PI-IBS have been shown to persist for up to 8–10 years following an episode of enterocolitis.[23] *Campylobacter* species, especially *C. concisus* and *C. showae*, were detected in ~40% IBD patients compared to 13% in non-IBD controls.[24] Reactive arthritis, a spondyloarthopathy predominantly affecting knees, ankles and feet, can develop in 3–5% individuals following *Campylobacter* infection among other gastrointestinal and genitourinary infections in humans.[25] These sequelae can result in significant impairment in quality of life and health-care utilization. A particularly devastating complication following *Campylobacter* enterocolitis is Guillain-Barre syndrome (GBS) characterized by muscle weakness, respiratory distress or ascending paralysis.[26] Although it is a rare sequela (0.1–0.02%), up to 40% of GBS cases in the U.S. are triggered by *Campylobacter* enterocolitis, making *Campylobacter* infection the most frequently identified predisposing factor for GBS.[25, 27] Failure of quick recognition can result in prolonged paralysis or even death from this complication.

Clinical diseases and treatment options in animals

Campylobacter is widely distributed in various animal species. In most species, it exists as an intestinal commensal without causing clinical diseases, but it may induce localized enteritis or systemic infections in some circumstances. Reproductive losses (e.g. abortion and infertility) in ruminants are among the most significant clinical conditions associated with *Campylobacter* infection in animals. *C. jejuni* and *Campylobacter fetus* subsp. *fetus* (CFF) are the primary *Campylobacter* species associated with outbreaks of sheep abortions worldwide, and they also cause sporadic abortion in cattle and goats.[28] Both organisms are frequently found in the intestine and gall bladder of healthy animals; however, in infected pregnant ewes translocation of *Campylobacter* across intestinal mucosa and systemic spread may occur, leading to fetoplacental infection and abortion, which typically happens in the third trimester of gestation.[29] Historically, CFF was the primary *Campylobacter* species associated with ovine abortions worldwide,[30, 31] but an etiological shift from CFF to *C. jejuni* occurred in the U.S. where the majority of *Campylobacter*-associated sheep abortions are now attributed to a single genetic clone of *C. jejuni*[32]. For prevention and control of *Campylobacter*-associated sheep abortion, vaccination is a common practice, but the effectiveness varies.[33] Tetracycline is frequently used for control of the disease, and more

recently tulathromycin has become an alternative treatment due to the concern with tetracycline resistance in *Campylobacter*.[34]

Infectious infertility, aka bovine genital campylobacteriosis, characterized by infertility, early embryonic deaths and to a lesser extent abortion, is caused by *C. fetus* subsp. *venerealis* (CFV) and is an economically important disease of cattle worldwide.[28] The bacterium lives in the genital tract of cattle and transmitted venereally to cows by carrier bulls.[29] Control and prevention includes the identification and removal of carrier bulls as well as vaccination and antimicrobial treatment of bulls and cows.[35, 36] Although vaccination is overall an effective control strategy, complete elimination of CFV from infected animals appears to be more challenging.[36, 37]

C. jejuni is commonly present in the intestinal tract of chickens as a commensal. However, a recently identified *Campylobacter* species, *Campylobacter hepaticus*, has been shown to cause spotty liver disease (SLD) around the world.[38] SLD manifests as acute infectious hepatitis and is characterized by many multifocal, small necrotic foci on the surface of the liver. It affects mostly free-range layer chickens with up to 15% mortality and 35% reduced egg production. Chlortetracycline has been used as a treatment option during outbreaks, and currently there are no commercial vaccines available for SLD.[39]

In addition to farm animals, companion animals (such as dogs and cats) may carry various *Campylobacter* species (primarily *C. upsaliensis* and *C. jejuni*) in their gastrointestinal tract asymptomatically, but *Campylobacter* occasionally causes enteritis in these species, especially in younger animals.[40] *C. jejuni* is also recognized as a rare cause of abortion in dogs.[41] In the U.S., a recent multistate outbreak of human illnesses caused by multidrug resistant *C. jejuni* strains was epidemiologically linked to contact with puppies in commercial pet stores,[7] illustrating the significance of dogs as a source of *Campylobacter* for human infections.

Antibiotic resistance and alternative antibiotic therapy

Campylobacter is exposed to antibiotics used in food producing animals, companion animals, and humans. The organism is highly adaptable to antibiotic selection pressure and has developed various antibiotic resistance mechanisms (see recent review articles[42–46]). The resistance to fluoroquinolones is especially a concern as in many countries, the majority of *Campylobacter* isolates are no longer susceptible to this class of antibiotics.[18, 47–50] In the U.S., a recent CDC report revealed a rising trend of ciprofloxacin-resistant *Campylobacter* for the past two decades and the resistance rate reached 29% in 2017.[9] A unique feature of fluoroquinolone resistance in *Campylobacter* is its continued persistence or even increased prevalence in the absence of antibiotic usage.[44] A recent example is a published study in Australia, where fluoroquinolones have never been used in poultry, but the rate of fluoroquinolone resistance in *C. jejuni* isolates of poultry origin has recently risen to almost 15%.[51] With regard to macrolide resistance in *Campylobacter*, the resistance rate remains low in the U.S and Europe,[52, 53] while high prevalence of macrolide-resistant *Campylobacter* has been reported in developing countries.[54–56] The recent emergence of *erm*(B), which encodes a rRNA methyltransferase and is able to confer a high-level

macrolide resistance (erythromycin MIC ≥ 256 μg/ml) in *Campylobacter*,[57–59] may further threaten the utility of macrolide antibiotics for clinical therapy.

In the regions where fluoroquinolone resistance is known to be highly prevalent, macrolide antibiotics (e.g. azithromycin) should be considered as the first line of antibiotics for therapeutic treatment of campylobacteriosis.[60] For systemic infection, aminoglycoside antibiotics, such as gentamicin, remain the therapeutic option as *Campylobacter* isolates are generally susceptible to this class of antibiotics; however, the recent emergence of novel aminoglycoside resistance genes and multidrug resistance genomic islands that confer resistance to multiple aminoglycoside antibiotics poses a threat to clinical utility of aminoglycoside antibiotics.[61, 62] Additionally, carbapenems were successfully used to treat *Campylobacter*-associated bacteremia and sepsis and were suggested as an alternative antibiotic for *Campylobacter*-associated systemic infections.[63, 64] Concerned with the rising resistance to fluoroquinolone and macrolide in *Campylobacter*, some investigators proposed the use of the amoxicillin-clavulanic acid combination as an alternative antibiotic therapy for campylobacteriosis.[18, 65] This proposition was supported by the evidence that *Campylobacter* isolates from pediatric patients and international travelers were uniformly susceptible to amoxicillin-clavulanic acid based on *in vitro* susceptibility tests.[18, 66] However, in a case report published by Aguilar-Comapany *et al.*,[67] amoxicillin-clavulanic acid failed to clear recurrent diarrhea in two patients infected by *Campylobacter* that was resistant to both fluoroquinolone and macrolide. Instead, fosfomycin tromethamine was successful in clearing the infection in both cases, suggesting that it could be used as alternative therapy for campylobacteriosis caused my multidrug-resistant *Campylobacter*. Additionally, Casagrande Proietti *et al.* reported that the majority of *C. jejuni* and *C. coli* isolates from chicken were resistant to amoxicillin-clavulanic acid.[68] Considering these findings, the clinical utility of amoxicillin-clavulanic acid in treating campylobacteriosis remains uncertain. Additional studies are necessary to examine alternative antibiotic therapies and to develop new approaches for control of *Campylobacter* infections.

New and non-antibiotic approaches to the control of *Campylobacter*

The increased concern with antibiotic resistance in *Campylobacter* has heightened research efforts in developing new and alternative control strategies for this pathogen. Since human campylobacteriosis cases are primarily contracted via the foodborne route, successful control of the disease requires mitigations in both animal reservoirs and the human host. To date, a number of studies have been attempted to reduce *Campylobacter* colonization in food producing animals, with the expected outcome of improving public health by reducing sources of infection. In this section, we will review various strategies that are being developed for controlling *C. jejuni and C. coli* both in humans and in food producing animals. The intention is to provide a broad overview on various approaches, instead of an in-depth evaluation of a particular strategy. Our perspectives and insights for future development are also provided when appropriate. Although other *Campylobacter* species (non- *C. jejuni*/*coli*) may also be associated with diseases in human and animals, they are less significant and little information on alternative control strategies is available for them. Thus, they will not be covered in this section.

Prebiotics.

The International Scientific Association for Probiotics and Prebiotics recently defined prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[69] Some well-known examples of prebiotics are inulin, galactooligosaccharides, and fructooligosaccharides (FOS). Human milk oligosaccharides (HMOs) are considered as natural prebiotics and play an important role in shaping beneficial microbiota in the intestine of infants.[69] In addition to improving intestinal health, HMOs have been shown to directly block *C. jejuni* attachment to host cells and inhibit *Campylobacter* colonization in a mouse model.[70, 71] This could be explained by the finding that *Campylobacter* binds to H-2 antigen on intestinal epithelial cells, while HMOs inhibits the binding of *Campylobacter* to the H-2 antigen. A recent study further indicated that a fucosylated HMO significantly reduced *C. jejuni* invasion into cultured Hep-2 and HT-29 cells and decreased the release of proinflammatory cytokines *in vitro*.[72] These experimental findings were corroborated by evidence from an epidemiological study, in which high-level of fucosylated HMO was found to be correlated with protection against *Campylobacter*-induced diarrhea in breast-fed infants.[73] Practical application of HMOs as prebiotics requires large-scale production. Interestingly, Weichert *et al.* demonstrated the feasibility of using genetically engineered *E. coli* to produce biosynthesized HMOs (2'-fucosyllactose and 3-fucosyllactose) and found that the synthesized 2′-fucosyllactose reduced *Campylobacter* adherence to *in vitro* cultured Caco-2 cell at a level comparable to human breast milk.[74] This suggests that HMOs produced by bioengineering methods may have similar functions as those found in human breast milk. Future studies using animal models or clinical trials are needed to examine whether biosynthesized HMOs can be used effectively for the control of *Campylobacter* infection in humans.

As an alternative for antibiotics, prebiotics have also been studied for their use to prevent and reduce *Campylobacter* colonization in animals, especially in broiler chickens.[75] However, the findings were inconsistent. For example, one study found that mannan-oligosaccharide, when provided as feed supplement at 0.2%, significantly reduced *Campylobacter* numbers in cecal contents of chickens and litter samples.[76] In another study, feed supplemented with 1% inulin or 1% oligofructose significantly decreased *Campylobacter* colonization in the large intestine, but not in the gizzard and small intestine.[77] In contrast to the results described above, several studies on prebiotic or prebiotic-like treatments did not reveal any significant effects on *Campylobacter* counts in broiler chickens.[78, 79] Together, these results suggest that prebiotic effects on *Campylobacter* colonization are variable and may not be consistently reproduced, posing a major challenge for practical use of prebiotics to control *Campylobacter* in animal reservoirs. Currently, there is little understanding of how prebiotics modulate the interaction between *Campylobacter* and the intestinal microbiome and how the interaction influences the outcomes of *Campylobacter* colonization. Future research efforts in these directions are needed, which may generate useful information for the development of prebiotic-based strategies for mitigating *Campylobacter* colonization in food producing animals.

Probiotics.

Probiotics are living non-pathogenic organisms that produce beneficial effects on hosts.[80] To the best of our knowledge, there has been no published work on the use of probiotics for mitigating *Campylobacter*-associated infection or disease in human. However, several studies have been conducted using cell cultures or mouse models.[81–84] These studies demonstrated probiotic products, such as *Bacillus* and *Lactobacillus*, reduced *Campylobacter* colonization in mice, *C. jejuni* invasion into cultured human epithelial cells, or release of pro-inflammatory cytokines from *Campylobacter*-infected cells. Since chicken is a major reservoir for *Campylobacter*, there have been active efforts in developing probiotics to reduce *Campylobacter* colonization in poultry. Probiotics made of *lactobacilli* inhibited *C. jejuni* growth culture media and reduced *Campylobacter* colonization in broiler chickens.[85] Some probiotic bacterial isolates (e.g. *Bacillus* and *Lactobacillus* spp.) derived from the ceca of healthy birds significantly decreased the level of *Campylobacter* colonization in chickens.[86] Additionally, a probiotic product made of *L. acidophilus* and *Streptococcus faecium* not only decreased colonization but also reduced shedding of *C. jejuni* in chickens.[87] A more recent study found that a probiotic made of *L. johnsonii* altered the gut microbiota and reduced *Campylobacter* colonization in ceca of chickens.[88] Despite these reported beneficial outcomes of probiotics, there are also multiple published studies that did not demonstrate an antagonistic effect on *Campylobacter* colonization in the poultry host.[89–91]

In order to be effective, probiotics must be able to establish in the intestinal tract of inoculated birds. Therefore, the efficacy of probiotics may be affected by factors that influence the establishment, such as the ability to survive low pH in the gastric environment, doses of probiotics, and the route of administration. For example, a study by Arsi *et al.* evaluated the efficacy of 10 probiotic isolates by using two different routes of inoculation: oral or intracloacal.[92] The authors found that only one of the 10 probiotic strains yielded a 1 log unit reduction in *Campylobacter* counts in ceca when they were given orally; however, six of the 10 probiotic strains decreased cecal *Campylobacter* counts by 1–3 log units when they were given intracloacally. Although the intracloacal route of inoculation may not be a practical way for on-farm application, the results indeed suggest the need for improved delivery of probiotics into the intestinal tract to increase their efficacy against *Campylobacter*.[92] In general, the efficacy of probiotics has been primarily evaluated under experimental conditions, which may not be applicable to the production environments on poultry farms. Additionally, the exact mechanisms by which probiotics inhibit *Campylobacter* colonization are understudied, hindering the development of probiotics that produce consistent and reproducible results. With the advance of new technology, now it is possible to study the complex interactions among *Campylobacter*, probiotics, gut microbiome, and the host. These research efforts should guide the targeted development of effective and reliable probiotics in the future.

Fatty acids.

Fermentation of undigested polysaccharides by gut anaerobes produces short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA).[93] These fatty acids not only serve as energy sources for the gut epithelial cells, but also have anti-*Campylobacter* activities. Thus,

SCFA and MCFA have been evaluated as feed additives for inhibiting *Campylobacter* establishment in poultry. Van Deun *et al*. examined several SCFAs using experimental systems and found that butyrate was the most efficacious SCFA against *C. jejuni* in culture media; however, it failed to reduce *C. jejuni* colonization in broiler chickens when given as a feed supplement.[94] In contrast, Guyard-Nicodème *et al.* found that several SCFA-based feed additives reduced *Campylobacter* colonization in broiler chickens compared to the non-supplemented feed, although the effect did not last for the entire experimental period (42 days) for some of the SCFA-based products.[95] Solís de los Santos reported that when caprylic acid (a MCFA) was given to broiler chickens as a feed additive for either 3 days or 7 days before they were slaughtered, it resulted in > 3 log unit reduction in *Campylobacter* counts compared with the birds on non-supplemented feed.[96] On the other hand, Hermans *et al.* found that although MCFAs showed robust killing activities against *Campylobacter* in culture media, they did not affect *Campylobacter* colonization in broiler chickens when given either in feed or in drinking water.[97, 98] These results clearly illustrated the variable effects of SCFA and MCFA on reducing *Campylobacter* colonization in different studies. In order to be effective, the concentration of these fatty acids must reach to the inhibitory level for *Campylobacter* in the chicken gut. Additionally, the complex environments in the intestinal tract may further undermine the action of SCFA and MCFA on *Campylobacter*.[97] These factors should be considered in future development of fatty acid-based applications.

Bacteriocin.

Bacteriocins are small peptides of bacterial origin that exhibit anti-bacterial activities by disrupting bacterial membrane.[99] It was estimated that 30–90% of bacterial species make at least one bacteriocin.[100] Many bacteriocins are produced by commensals in intestine, providing a competitive advantage to the commensal bacteria and functioning as an innate defense mechanism against pathogenic organisms.[101, 102] Bacteriocins are considered a potential alternative for antibiotics,[103, 104] and have been explored for mitigating *Campylobacter* in chickens.[105] Stern *et al.* reported that a bacteriocin (named SRCAM 602) isolated from *Paenibacillus polymyxa* produced more than 7 log unit reduction in *Campylobacter* colonization in chickens when given in feed.[106] The finding that *Campylobacter* was not detectable in any of the bacteriocin-treated chickens suggested that SRCAM 602 might be used as a therapeutic agent to eliminate *C. jejuni* from chickens. Subsequently, the same team described bacteriocins OR-7, E-760, and E 50–52, which were isolated from *Lactobacillus salivarius* and *Enterococcus* sp., respectively, and in each case, the bacteriocin treatment resulted in drastic reduction of *C. jejuni* colonization in chickens compared to the non-treated controls.[107–109] Despite these highly promising findings, there have been no follow-up studies on application of these bacteriocins since 2011. In fact, there have been few published anti-*Campylobacter* bacteriocin studies for the past decade. Some recent examples include bacteriocins produced by *Lactobacillus salivarius* SMXD51 and *Lactobacillus curvatus* DN317.[110, 111] Both bacteriocins demonstrated good anti-*Campylobacter* activity *in vitro*, although their mode of action was different, with bacteriocin DN317 being bacteriostatic and bacteriocin SMXD51 being bactericidal. Despite the fact that they are effective against *Campylobacter in vitro*, whether they can effectively reduce *Campylobacter* colonization in chickens remains unknown. In general, the utility of bacteriocins as a therapeutic agent for *Campylobacter* treatment requires further

investigation. Particularly, the *in vivo* efficacy of various bacteriocins need to be verified and reproduced under natural poultry production conditions. Even if they are proven to be safe and effective, commercial use requires cost-effective production of bacteriocins in large quantities.

Bacteriophage.

As bacterial viruses, bacteriophages (phages) can infect and lyse bacterial cells. Phage infection of bacteria is determined by specific receptors on bacterial surfaces, such as outer membrane proteins, lipopolysaccharides and flagella components.[112, 113] Due to the rising concern with antimicrobial resistance, phage therapy has attracted renewed attention as a potential therapy to combat multidrug resistant bacterial pathogens including *Campylobacter* species.[113–117] There have been a number of studies on *Campylobacter* phages and their potential applications (see a most recent review[118] for detailed information). Many of these studies examined the efficacy of various phages in mitigating *Campylobacter* colonization in chickens. For example, Richards *et al.* used a mixture of two *Campylobacter* phages to treat chickens experimentally infected with *C. jejuni* and observed considerable reduction *in Campylobacter* counts in the intestinal tract throughout the 5-day treatment period, but the most obvious difference was seen 2 days after the initiation of the treatment.[119] Using experimentally infected broiler chickens, Wagenaar *et al.* demonstrated that phage therapy effectively decreased *Campylobacter* colonization when given either before *Campylobacter* inoculation as a preventive measure or after *Campylobacter* infection was established as a therapeutic approach.[117] The authors also noticed that the effect was most obvious for the first few days after the initiation of phage therapy. Similarly, Loc Carrillo *et al* demonstrated phage therapy reduced *Campylobacter* colonization in experimental chickens, and the levels of reductions varied with different phage-*Campylobacter* strain combinations, the phage dosages, and the time elapsed after phage administration.[115] To evaluate the efficacy of phage treatment under natural settings, Kittler *et al.* conducted three field trials using a cocktail of phages on broiler farms where the birds were naturally colonized by *Campylobacter*.[116] During the trials, the cocktail of phages was given to boiler chickens a few days prior to slaughter. Although trial 1 resulted in significant reductions (>3 log units) *in Campylobacter* counts in feces and cecal contents, trials 2 and 3 did not observe a significant difference between phage-treated groups and the non-treated controls.[116] This study illustrated the variable efficacy of even the same phage cocktail in different trials. A general observation from these phage therapy studies was the tendency for decreased efficacy over the course of treatment. This suggests that *Campylobacter* may be able to quickly adapt to phage treatment due to development of resistance or other reasons. Since reducing *Campylobacter* counts in the intestinal tract of chickens destined for slaughter will lead to less carcass contamination in the slaughtering process, phage therapy may be potentially used as a treatment right before slaughter to reduce the risk of *Campylobacter* transmission via contaminated chicken meat to consumers. Considering that bacteriophages tend to have strain specificities and a single poultry farm may harbor multiple different *C. jejuni* strains, practical applications should consider use of phage cocktails with broad activities against different *Campylobacter* strains.

Immunization.

There have been active efforts in developing vaccines as a preventive measure to control *Campylobacter* infections in humans and animal reservoirs. For human use, various vaccine candidates, such as killed whole cells vaccines, subunit vaccines, and capsule polysaccharide conjugate vaccines, have been investigated,[120–122] but many of these vaccine efforts were abandoned due to safety concerns or lack of efficacy in human clinical trials.[123] For detailed information on human *Campylobacter* vaccine development, we refer readers to a recent update by Poly and co-authors.[123] Currently there are no commercial vaccines on the market for human use. On the contrary, commercial vaccines have been utilized to control *Campylobacter*-induced infertility and abortion in cattle and sheep. These vaccines are inactivated whole cell bacterins made of multiple *Campylobacter* spp. or strains and may not be protective against the currently most prevalent *Campylobacter* strains.[124] Given the importance of poultry meat in transmitting *Campylobacter* to humans, a number of studies have been conducted to develop vaccines against *Campylobacter* colonization in broiler chicken, but most of the published work yielded limited success. A notable advance was the recent development of experimental glycoconjugate vaccines that were constructed by fusing the conserved *C. jejuni* N-glycan to a carrier protein or by linking it to the lipopolysaccharide core of *E. coli*.[125, 126] The vaccines induced IgY antibodies that specifically recognized the N-glycan and demonstrated high efficacy in preventing *Campylobacter* colonization in both layer chickens and broiler chickens. Since the vaccines is made of a conserved glycan, they are expected to provide broad protection against different *C. jejuni* strains. This remains to be determined by field trials on commercial farms where chickens are naturally colonized by genetically and antigenically diverse *Campylobacter* strains.

Passive immunization, i.e. oral administration of hyperimmune antibodies as a prophylactic or therapeutic agent, has been evaluated as a potential approach for preventing or reducing *Campylobacter* colonization in chickens. Laying hens naturally infected by *Campylobacter* or hyperimmunized with *Campylobacter* antigens produce high-titer anti-*Campylobacter* antibodies that are transferred to egg yolks, as mean to transfer maternal antibodies from layers to young hatchlings. Egg-derived maternal antibodies (IgY) were shown to protect, at least partially, young chickens from *Campylobacter* colonization.[127] Several studies explored the feasibility of the passive immunization approach and demonstrated that hyperimmune egg yolk antibodies, when given to chickens orally or as feed supplements, produced significant reduction in *campylobacter* colonization in the intestine.[128–130] The effect was especially obvious when hyperimmune egg yolk antibodies were given prophylactically (before *Campylobacter* inoculation), although significant reduction was also observed with therapeutic use (i.e. administered to chickens after *Campylobacter* infection was established). Additionally, antibodies induced by whole cell vaccines produced better protection than antibodies generated by subunit vaccines made of selected proteins from *C. jejuni*.[128, 129] However, in the study by Paul *et al.*, it was found that hyperimmune egg yolk antibodies generated by immunizing hens with subunit vaccines did not affect *Campylobacter* colonization in chicken ceca when given as feed supplements.[131] This discrepancy might be due to the fact that different antigens were used in the subunit vaccines, which might not be able to generate protecting antibodies against colonization.

Overall, these studies demonstrate the potential of passive immunization and suggest that the protection is influenced by the antigens used to prepare the hyperimmune egg yolk antibodies. Antigen selection is especially important considering *C. jejuni* strains are antigenically diverse and there are many different strains existing in nature.

Recently, nanobodies have been explored as a potential mean to control *Campylobacter*. Unlike the conventional antibody that contains both heavy chains and light chains, nanobodies produced by camelids lack light chains and carry only a single antigen-binding domain of the heavy chain.[132, 133] Nanobodies are small in size and can be easily produced as recombinant proteins. Additionally, nanobodies are stable and have good tissue penetration properties.[132] These unique features make nanobodies ideal candidates for development of various therapeutics.[133, 134] In one study, Vanmarsenille *et al.* successfully produced six nanobodies that recognized surface-exposed epitopes of the major outer membrane protein (MOMP) in *Campylobacter* and showed a broad reactivity with different *C. jejuni* and *C. coli* strains.[135] Notably, all 6 nanobodies were found to preferably react with native MOMP, and nanobody-coated beads agglutinated *Campylobacter* cells, indicating they are functionally active in recognizing surface epitopes. Recently, the same team made chimeric antibodies by fusing nanobodies recognizing *Campylobacter* MOMP and flagellin with the constant domains of IgY and IgA of chicken, and successfully expressed the chimeric antibodies in plant leaves and seeds.[136] The plant produced antibodies showed binding activities to native MOMP and intact *Campylobacter* cells, and the plant-derived flagellin-specific antibodies reduced the motility of *Campylobacter*. These results demonstrate potential use of genetically engineered nanobodies for control of *Campylobacter* infection. However, the efficacy of anti-*Campylobacter* nanobodies has not been examined in animal models and their utility as a potential therapeutic approach remains to be investigated in future studies.

Antibiotic adjuvants.

One approach to combating antibiotic-resistant pathogens is to resensitize them to currently available antibiotics by using antibiotic adjuvants,[137] which by themselves are not antibacterial but can augment the activities of antibiotics when both are combined. For the purpose of developing antibiotic adjuvants against *Campylobacter*, the CmeABC multidrug efflux pump is a promising target as it is the primary antibiotic efflux system in *Campylobacter* and is a critical player in the resistance to different classes of antibiotics.[138] CmeABC also mediates bile resistance in *Campylobacter* and is required for *Campylobacter* to survive and grow in animal intestine.[139] Thus, inhibition of CmeABC should increase antibiotic accumulation in *Campylobacter* and enhance its susceptibility to antibiotics. Two possible strategies have been examined to inhibit this efflux pump in *Campylobacter*: interfering with extrusion by efflux pump inhibitors (EPIs) and inhibiting expression by antisense peptide nucleic acids (PNAs).[140, 141] EPIs are small molecules that can interact with an efflux transporter and consequently “clog” the extrusion of antibiotics. For *Campylobacter*, two EPIs, phenyl-arginine-β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP), have been evaluated for inhibition of antibiotic efflux.[142–144] A general observation from studies in different laboratories was that PAβN was fairly effective in potentiating macrolide antibiotics, but had little effect on fluoroquinolones,

while NMP was much less effective than PAβN in potentiating antibiotics. Plant extracts have also been used to modulate antibiotic activities against *Campylobacter*. For example, Oh and Jeon found that several phenolic compounds sensitized various *C. jejuni* isolates to ciprofloxacin and erythromycin considerably and the authors postulated that the synergizing effect of the phenolic compounds with antibiotics was possibly due to reduced antibiotic efflux and increased membrane permeability in *Campylobacter* cells.[145] In a recent study by Klancnik *et al.*, it was reported that extracts of *Alpinia katsumadai* seeds modulated antibiotic efflux activity in *Campylobacter* and reduced MICs of various antibiotics including erythromycin and ciprofloxacin.[146] Whether the plant extract functions as a natural EPI for CmeABC remains to be determined.

PNAs are synthetic polymers of DNA mimics, bind to nucleic acids with high affinity and specificity, and are resistant to proteases, nucleases, and low pH.[147] These characteristics have made PNA a useful mean for antisense inhibition of gene expression in various bacterial organisms.[148, 149] Different from EPIs, PNAs don't directly interact with efflux transporters. Instead, they are designed to target genes encoding multidrug efflux pumps and thereby inhibit their expression in bacterial cells. PNAs have been successfully used to inhibit *cmeABC* expression in *Campylobacter*.[140, 150] Specifically, various PNAs targeting the CmeABC operon reduced the expression of this efflux system and sensitized *Campylobacter* to ciprofloxacin and erythromycin in both wild-type and antibiotic resistant *C. jejuni* strains. It was further found that the PNA targeting the ribosome binding site of *cmeA* was the most effective in the inhibition of *cmeABC* expression.[140] These results suggest the potential of CmeABC-specific PNAs as an adjuvant for antibiotic therapy to combat antibiotic-resistant *Campylobacter*. The *in vivo* efficacy of PNA in potentiating antibiotics against *Campylobacter* are being evaluated in animal models. In addition to targeting *cmeABC*, PNAs may also be designed to target other antibiotic resistance determinants in *Campylobacter*, which has not been evaluated and remains to be explored in future studies. Currently, the PNA approach has two drawbacks. First, PNA itself is poorly permeable to bacterial membrane and use of PNA requires it to be conjugated to a cationic peptide for enhanced penetration. Secondly, PNA is expensive to produce, which is a major limiting factor for *in vivo* trials. Technological advance in improving PNA's cell permeability and reducing production cost should significantly enhance the utility of this antisense approach.

Conclusion Remarks

To date, multiple strategies have been evaluated to control *Campylobacter* infections in animal reservoirs and in the human host. Although some of them have yielded promising results, none of these alternative approaches are as effective as antibiotics in clearing *Campylobacter* infections. For antibiotic therapy in human patients, alternative antibiotics may be considered when *Campylobacter* is resistant to the first-line antibiotics, but additional studies in clinical settings are needed to identify the optimal alternatives. Additionally, further efforts should be directed to develop antibiotic adjuvants that may improve the utility of existing antibiotics. For vaccine development, some candidate vaccines showed good protective effects in experimental animal models (e.g. mouse or non-human primate), but they were not able to produce protective immunity in human clinical

trials, suggesting that host specificity plays important roles in immunization with *Campylobacter* vaccines.[123] Thus, more research is warranted to better understand mechanisms underlying *Campylobacter*-host interactions and the nature of protective immune responses in humans. These efforts should help identify novel targets and provide new directions for future vaccine development. Significant amount of research has been performed for mitigating *Campylobacter* colonization in animal reservoirs. This is particularly true with poultry as it serves as a major source of *Campylobacter* for human infections. Some approaches (such as the N-glycan based vaccine, bacterin, and phage therapy) have shown encouraging results, while others (e.g prebiotics and probiotics) are met with limited success as their effects are modest and highly variable. Significant improvements in efficacy and reliability are necessary to increase the utility of these alternative approaches in poultry production. Additionally, any products that are destined for use on poultry farms should be produced in a cost-effective manner as economic factors have a major impact on their practical applications in the field. To achieve the optimal outcome in the control of *Campylobacter* in the food chain, combination of multiple approaches may be necessary, and this possibility warrants further studies. Effective preventive and therapeutic interventions will not only reduce the diarrheal burden, but also have an impact on chronic sequelae due to *Campylobacter* infection and the associated medical costs for the health-care industry.

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